EXHIBIT 1

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RAPID COMMUNICATION

Measurement of Prostate-Specific Membrane Antigen in the Serum With a New Antibody

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ABSTRACT: Work to date has identified prostate-specific membrane antigen (PSMA) as a membrane-bound glycoprotein with high specificity for prostatic epithelial cells. PSMA reacts with the monoclonal antibody 7E11.C5, which is present in serum, seminal fluid, and prostatic epithelial cells, and is increased in its expression in the presence of a hormone refractory state associated with prostatic cancer. This report confirms these results and further documents the presence of the monoclonal antibody 3F5.4G6, which reacts with the extracellular domain of PSMA. This region of PSMA is also an element present in a truncated version of the protein, so-called PSM'. Immune precipitation with either 7E11.C5 or 3F5.4G6 yields an isolated protein species that are reactive with the reciprocal antibody in Western blot analysis. Thus, 3F5.4G6 recognizes the same PSMA protein as does 7E11.C5, but at different epitopes on essentially opposite ends of the molecule. These two antibodies are well suited for use in a sandwich immunoassay, either one as a capture or detection antibody. Current work on this is underway.

This report also confirms that 7E11.C5 Western blots for PSMA are negative with normal human brain tissue. The monoclonal antibody 9H10 does not react with 3F5.4G6 or with 7E11.C5 in studies conducted herein. Moreover, 3F5.4G6 reacts with PSMA found in the LNCaP cell line, but not DU-145 or PC3, which lack PSMA.

KEY WORDS: prostate-specific membrane antigen (PSMA), prostate cancer, prostate marker

INTRODUCTION

We have previously described an antibody 7E11.C5 that can, by enzyme-linked immunosorbent assay (ELISA) (with another antibody 9H10) or by Western blot, detect in prostate cancer patients a protein called prostate-specific membrane antigen (PSMA) [1–3]. To validate this observation further, we collected sera in a prospective multicenter study. The sera were run on a double-blind basis without knowledge of the clinical state, or the presence or absence of prostate cancer [4]. Furthermore, we compared levels of prostate-specific antigen (PSA) concurrently with PSMA values [4]. The elevated PSMA levels predicted a state of clinical progression or clinical resistance in most cases (>70%). PSMA levels were of better prognostic value than PSA [4]. Despite these observations, there have recently been publications questioning whether PSMA was present in the serum [5,6]. We have completed a series of experiments which validate our original observations, and herein report such.

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MATERIALS AND METHODS

Cell Lines and Reagents

P3X63Ag8U.1 (X63), an HPGRT-negative mouse myeloma cell line (CRL 1597 from ATCC, Rockville, MD), was maintained in 90% RPMI-1640: 10% Fetal Clone (HyClone, Logan, UT) supplemented with 2 mM L-glutamine, penicillin/streptomycin, and 1 mM sodium pyruvate. Rabbit antimouse IgM and antimouse IgG were purchased from ICN (Costa Mesa, CA). Peroxidase-labeled goat antimouse IgG and goat antimouse IgM were purchased from Kirkegaard and Perry Laboratories (KPL, Gaithersburg, MD). LNCaP, a prostate cancer cell line (CRL 1740, from ATCC, Rockville, MD) expressing PSMA, was maintained in 95% RPMI-1640/5% fetal calf serum (FCS).

Preparation of Immunogen and Immunization of Mice

PSMA-derived peptide 716–723 (NH_2-ESKVDPSK−) was coupled to keyhole limpet hemocyanin (KLH) as a carrier protein using the EDC coupling method of Pierce (Rockford, IL). The peptide–carrier complex was emulsified in incomplete Freund's adjuvant (Sigma, St. Louis, MO) containing 1 mg/ml muramyl dipeptide (MDP, Pierce) at a final concentration of 250 μg/ml. BALB/c mice were immunized subcutaneously with 100 μl of the emulsified peptide–carrier complex every 2 weeks. Following the third injection, blood was obtained from the mice and their sera were tested for antipeptide antibodies in a peptide-specific radioimmunoassay (RIA). Spleens from donor mice demonstrating an antipeptide titer of 1:1,000 or greater were used in a fusion protocol with X63 myeloma cells.

Fusion Protocol and Initial Screening for Antipeptide Producing Hybridomas

Three days prior to fusion, the donor mouse was immunized intraperitoneally with 50 μg of peptide–carrier complex in saline. The spleen was aseptically removed, and a single cell suspension was prepared in RPMI-1640 medium without serum. The splenocytes were added to X63 myeloma cells at a ratio of 10:1, and the fusion was performed by the method of Galfré and Milstein [7]. Following fusion, the splenocyte–myeloma mixture was resuspended in 80% RPMI-1640/20% Fetal Clone supplemented with aminopterin, to act as a selective medium for hybridoma growth and plated in 200-μl volumes into sterile 96-well microtiter plates.

Ten to 14 days following fusion, 50 μl of cell culture supernatant from the individual wells was removed and tested in an RIA for peptide-specific antibodies. Briefly, the supernatants were added to wells of a Pro-Bind plate (Falcon) coated with PSMA-peptide coupled to bovine serum albumin (BSA) by the EDC method referenced above and blocked with BSA. Following an overnight incubation at 4°C the plates were washed 4 times with PBS-0.1% BSA. Fifty μl of a 1:500 dilution of rabbit antimouse IgM and antimouse IgG was added to each well, and the plates were incubated for 1 hr at room temperature. Following four washes, 50 μl of ^{125}I-Protein A (≈10^5 cpm/well) was added to each well and the plates incubated for an additional hour at room temperature. The plates were washed four times and exposed to X-ray film (Kodak, X-OMAT) overnight and developed. Positive wells showing antipeptide-specific antibodies were identified from the exposed film and the cells from the positive wells were expanded in 90% RPMI-1640/10% Fetal Clone for further testing.

Western Blot Analysis of Primary Hybridomas for anti-PSMA Antibody

Supernatants from the expanded antipeptide-reactive primary hybridomas were tested in a Western blot assay for the presence of anti-PSMA antibodies. Western blot analysis was performed following the protocol of Pelletier and Boynton [8]. Briefly, lysates from LNCaP cells, a prostatic tumor cell line that expresses PSMA, were electrophoresed on an 8.5% gel by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were electroblotted onto a PVDF membrane for 1 hr at 90 V. The membranes were blocked overnight in 5% milk–TBS. The blocked membrane was placed in a multiscreen apparatus (BioRad), and approximately 650 μl of hybridoma supernatant was pipetted into individual lanes. Following a 90 minute incubation at room temperature the blot was removed from the apparatus, washed five times for 5 min in TBS–0.5% Tween-20 (TBS-T), and probed with 167 ng/ml of peroxidase-labeled goat antimouse IgG secondary antibody (KPL, Gaithersburg, MD) for 30 min at room temperature. The membrane was washed five times for 5 minutes with TBS-T, and the membrane was developed using the Chemiluminescent Substrate Kit (KPL). The blot was visualized by exposing X-ray film. Positive hybridomas (anti-PSMA reactivity) were identified and selected for further development.

Cloning by Limiting Dilution, Testing Clones, and Purification of Monoclonal Antibody

Primary hybridomas identified by their anti-PSMA reactivity were cloned by limiting dilution. Briefly, the cells were adjusted to a final concentration of 1 cell/ml of RPMI-1640–10% Fetal Clone containing 10^5 syngeneic thymocytes as a feeder cell population. Two hundred μl of cell suspension was pipetted into wells of

sterile 96-well microtiter plates and cultured for 7–10 days, or until single colonies of cells were visible. Wells containing single colonies were picked, and the clones were expanded in 24-well plates. Supernatants from the clonal cultures were harvested and tested for anti-PSMA reactivity in the Western blot assay described above. Clones producing anti-PSMA monoclonal antibody were expanded, and the cells were used for the generation of high titer ascites fluid. The monoclonal antibody 3F5.4G6, an IgM class anti-PSMA antibody, was purified from ascites fluid using the ImmunoPure IgM Purification Kit (Pierce, Rockford, IL).

Immunoprecipitation of PSMA from LNCaP Tumor Cells Using 3F5.4G6 Monoclonal Antibody

Approximately 10×10^6 LNCaP tumor cells were incubated with 1 ml of NP-40 lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, pH 8.0) for 30 min at 4°C. The lysate was centrifuged at 12,000 rpm for 5 min to remove cellular debris and the resultant supernatant was precleared by incubation with 50 μl of normal mouse serum (Sigma) for 30 min followed by the addition of 60 μl of a 20% suspension of antimouse IgM-coupled agarose beads (Sigma). Following 1-hr incubation at 4°C, the supernatant was centrifuged at 12,000 rpm to remove the beads, the resultant supernatant was used in an immunoprecipitation protocol with 3F5.4G6 monoclonal antibody. Ten μg of purified 3F5.4G6 monoclonal antibody was added to the supernatant and incubated for 1 hr at 4°C. One hundred μl of a 10% suspension of antimouse IgM-coupled agarose beads was added, and the supernatant was incubated for an additional hour at 4°C. The samples were centrifuged at 12,000 rpm, and the agarose beads were washed three times with NP-40 lysis buffer. The agarose beads were resuspended in 30 μl of sample buffer (SDS reducing buffer) and heated for 10 min at 95°C. Following a brief centrifugation at 12,000 rpm, the sample was run on an 8.5% SDS–PAGE gel, and the separated proteins were electroblotted as described above. A Western blot assay as described above was performed on the samples using the PSMA-specific monoclonal antibody 7E11.C5 as the reporting antibody.

In additional controlled studies, the 9H10 monoclonal antibody was employed. This antibody reacts with an unknown protein on the surface of only LNCaP cells as was initially described, in contrast to 7E11.C5, which reacts to prostate epithelial cells [3].

RESULTS AND DISCUSSION

PSMA is a membrane-bound glycoprotein that shows high tissue specificity for prostatic tissues.

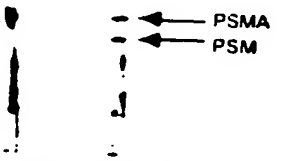
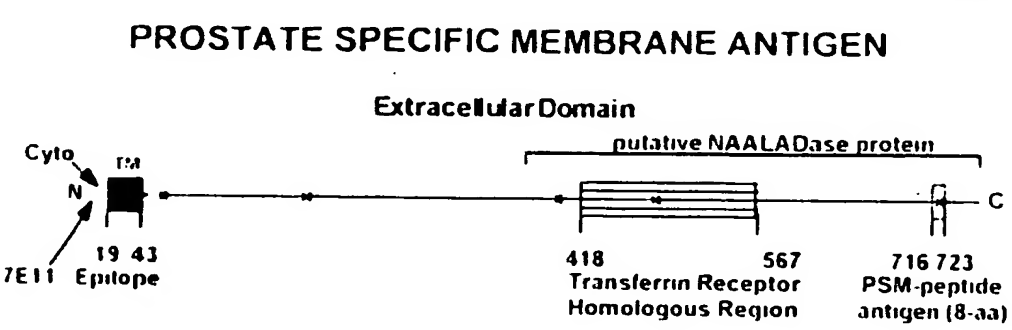


Fig. 1. Western blot assay of LNCaP lysate using 7E11.C.5 (*lane 1*) and 3F5.4G6 (*lane 2*) monoclonal antibodies and developed with HRP-anti-IgM secondary antibody. It should be noted that 3F5.4G6 recognizes a protein of M_r 120 kDa, which is similar, if not identical, to the protein recognized by 7E11.C5. 3F5.4G6 monoclonal antibody also recognizes a protein of M_r 110 kDa corresponding to the protein PSM'. It should be noted that 7E11.C5 does not recognizePSM' because the epitope of 7E11.C5 monoclonal antibody is N-terminal (amino acid 1'7) and is not found in PSM' because PSM' is a truncated version of PSMA and does not contain the initial 57 amino acids of PSMA. Thus, 3F5.4G6 recognizes the C terminal portion of the protein (amino acid 716–723) and specifically the extracellular domain of PSMA and PSM'.

PSMA was originally defined and its tissue expression characterized based upon the reactivity of the monoclonal antibody 7E11.C5 [3]. These results indicated high specificity for prostatic tissues and an increased expression of the 7E11.C5 antibody in the serum of prostatic cancer patients compared to normal individuals [2,3]. Further studies have confirmed this observation [1,4]. Other studies suggest that PSMA expression in tumors is down-regulated by steroids such as 5α-dihydrotestosterone [9,10]. This behavior is consistent with the elevated expression of PSMA in hormone-refractory tumors. Thus, the results indicate the antigen are almost entirely prostate specific in humans; furthermore, they may be a marker for aggressive clones of prostate cancer cells due to its increased expression in hormone-resistant tumors [1–4]. Figure 1 illustrates on Western blot the reactivity of 7E11.C5 with PSMA in LNCaP cells. It should be noted that monoclonal antibody 9H10 does not recognize a protein (i.e., PSMA) of M_r 110–120 kDa in LNCaP cells, but rather recognizes a protein of M_r 30 kDa of unknown identity.

Using the 7E11.C5 antibody as a probe, Israeli et al. [9] cloned a cDNA from LNCaP cells that encodes a 750-amino acid membrane protein. The deduced amino acid sequence defines a type II transmembrane protein composed of a short cytoplasmic domain, a membrane-spanning domain, and an extracellular domain, a portion of which has high sequence homology to transferrin receptor [9]. Further work has shown that the protein epitope to which the 7E11.C5 antibody binds is composed of the N-terminal amino acids of the protein located in the cytoplasmic domain [5,6]. That is, a "sixmer" composed of the first six amino acids from the N-terminal of PSMA was the



HOPP & WOODS ANTIGEN PREDICTION

AMINO ACID POSITION	SEQUENCE
479 - 486	K E L K S P D E
404 - 414	K K E G W R P R R
183 - 191	K E L K S P D E G
63 - 69	D E L K A E N
716 - 723	E S K V D P S K

Fig. 2. Topographical representation of PSMA regions in relation to Hopp and Woods antigen prediction. A recent paper reported a rat brain partial cDNA clone of NAALADase which possesses a high degree of identity to the 3' end of the PSMA cDNA. The putative NAALADase protein is highly homologous to a region of the extracellular domain of PSMA.

smallest peptide element recognized by 7E11.C5. No binding to any other peptide element not containing this terminal sequence was reported [5,6].

A variant of PSMA resulting from alternative splicing has been deduced from reverse transcriptase polymerase chain reaction (RT-PCR) studies and RNase protection assays [9]. This variant, designated PSM', is missing the first 57 amino acids of PSMA and was reported to predominate over PSMA in normal prostatic tissues while the reverse relationship was true in the case of prostatic carcinomas [10]. Thus, the PSM' protein is not recognized by the 7E11.C5 antibody (Fig. 1), and data supporting the physiological expression of PSM' to date rely solely on studies of the nature of mRNA species expressed in prostatic cells and tissues.

We have been interested in expression of PSMA as a possible marker for disease progression, particularly in later stages of the disease. Efforts have so far focused on development of serodiagnostic assays relying on the 7E11.C5 antibody for detection in a quantitative Western blot assay with serum. We now report, herein, the preparation of a monoclonal antibody designated 3F5.4G6 that is specific for an 8-amino acid region located near the C-terminal portion of the molecule (aa 716–723). An analysis of antigenic determinants based upon the Hopp and Woods algorithm demonstrated the antigenic potential of this protein region [11] (Fig. 2). Its presence in the extracellular domain near the C-terminal of the protein makes this determinant ideal for application to a sandwich-type immunoassay or to detect the presence of PSM' in the serum.

The results presented indicate that the 3F5.4G6 monoclonal antibody reacts specifically with the same protein species as 7E11.C5 in Western blots with LNCaP cell crude lysates (Fig. 3). Immune precipitation with either 7E11.C5 or 3F5.4G6 yields an isolated protein species, which in both cases is reactive with the reciprocal antibody in Western blot analyses (Fig. 3). Thus, it can be concluded that the 3F5.4G6 antibody recognizes the same PSMA protein as does 7E11.C5, but at different epitopes on essentially opposite ends of the molecule. The 3F5.4G6 antibody is thus well suited for use in a sandwich immunoassay as either a capture or detection antibody paired with the 7E11.C5 antibody. Current work is focused on this strategy.

The 3F5.4G6 antibody, in addition to its binding to the PSMA protein, cross-reacted with a protein species which correlates in size with the PSM' variant present in Western blots of LNCaP cell lysates (Fig. 3) by virtue of its specificity for a common epitope in the extracellular domain of both protein forms [10]. This, for the first time, provides direct evidence of the expression of PSM' at the protein level and provides a possible means for differential quantitation of each protein form in serum. 3F5.4G6 antibody also recog-

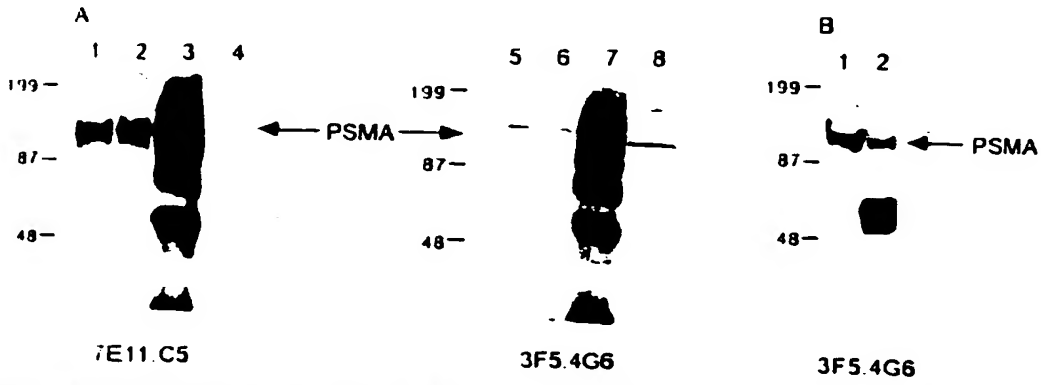


Fig. 3. **A:** Demonstration that the 7E11.C5 and 3F5.4G6 monoclonal antibodies recognize identical proteins and that 3F5.4G6 recognizes an additional protein corresponding to PSM'. LNCaP lysate was initially immunoprecipitated with 7E11.C5 monoclonal antibody and the immunoprecipitated material separated on SDS gels and probed in a Western blot assay with either 7E11.C5 (*lanes 1–4*) or with 3F5.4G6 (*lanes 5–8*) monoclonal antibodies. *Lanes 1, 5*, crude LNCaP lysate (0.05 μg protein/well); *lanes 2, 6*, precleared LNCaP lysate (0.05 μg protein/well); *lanes 3, 7*, material that immunoprecipitated with 7E11.C5 monoclonal antibody; *lanes 4, 8*, proteins left in the previously immunoprecipitated LNCaP lysate. It should be noted that 7E11.C5 immunoprecipitated a protein of M_r 120 kDa that was recognized not only by 7E11.C5 (*lane 3*), but also by 3F5.4G6 (*lane 7*). It should also be noted that present in the supernatant after 7E11.C5 immunoprecipitation was a protein recognized by 3F5.4G6 (*lane 8*), but not by 7E11.C5 (*lane 4*), and that corresponds to PSM'. Thus, 7E11.C5 does not recognize PSM' (i.e., the epitope of 7E11.C5 is amino acids 1–7 of PSMA while the epitope of 3F5.4G6 is amino acid 716–723) and therefore PSM' should remain in the lysate of a 7E11.C5 immunoprecipitated LNCaP lysate and subsequently recognized by 3F5.4G6. **B:** Demonstration that monoclonal antibody 7E11.C5 and monoclonal antibody 3F5.4G6 recognize identical proteins. PSMA from an LNCaP lysate was immunoprecipitated by monoclonal antibody 3F5.4G6, the proteins in the immunoprecipitate separated on a SDS gel, the proteins transferred to Immobilon P and probed in a Western blot with monoclonal antibody 7E11.C5. *Lane 1*, LNCaP lysate control; *lane 2*, 3F5.4G6 immunoprecipitation of 2.5 μg LNCaP lysate and Western blot with 7E11.C5 monoclonal antibody.

nized PSMA in the serum of prostate cancer patients (stage D2; Fig. 4), illustrating its utility in detection of PSMA in the serum of prostate cancer patients.

Studies of the expression of PSMA in the serum of both normal individuals and prostatic cancer patients has provided conflicting results at two laboratories [1–6]. Troyer et al. [5,6] reported evidence suggesting that the binding of 7E11.C5 to Western blots of serum specimens was nonspecific in that the peptide containing the antibody epitope (N1–19) was ineffective in inhibiting antibody binding to a protein species migrating in the same region as PSMA. These studies used differing conditions than was previously reported by this laboratory wherein serum expression was demonstrated [1–4]. The results presented in Figure 5 using the procedures we originally described indicate that 7E11.C5 binding to Western blots of both LNCaP cell lysates and human serum is specific and not due to nonspecific binding of secondary antibody. No labeling of any protein band corresponding to PSMA was detected in the absence of the 7E11.C5 antibody, even after extended exposure of the blot to X-ray film. This evidence strongly supports the presence of PSMA in human serum and is consistent with our earlier results demonstrating elevations in serum PSMA in prostatic cancer patients in clinical progression. In our results, we have also failed to demonstrate PSMA protein in normal human brain tissue, in contrast to a report by Troyer and colleagues, which has

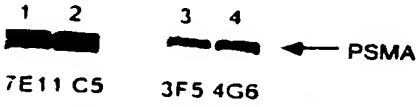


Fig. 4. Demonstration by Western blot of PSMA in serum of prostate cancer patients (stage D2) using monoclonal antibody 7E11.C5 (*lanes 1, 2*) and demonstration of the recognition of PSMA in the same prostate cancer patient by monoclonal antibody3F5.4G6 (*lanes 3, 4*). 0.44μg serum protein was loaded in each well and protein separated on SDS gels as described in Materials and Methods.

the same methodological limitations noted in Figure 5 [5,6]. In addition, studies using the 9H10 monoclonal antibody have failed to show that it interacts with 3F5.4G6 or 7E11.C5 antigen [3].

Moreover, 3F5.4G6 reactivity was not detected in DU-145 or PC3 cell lines—only in LNCaP, as is the case with 7E11.C5 [3]. There is, however, a most recent report demonstrating a clear nucleotide sequence identity for a region of the extra-cellular portion of PSMA (i.e., in the PSM' distal region) which possesses properties of NAALADase membrane hydrolase (figure 2).[12] We are conducting further work to clarify the molecular characteristics of the NAALADase region to 3F5.4G6 and 7E11.C5. Nevertheless, the availability of the 3F5.4G6 antibody for application in a sandwich immunoassay will simplify the detection of serum PSMA and provide a potentially useful clinical tool for monitoring prostatic cancer patients.

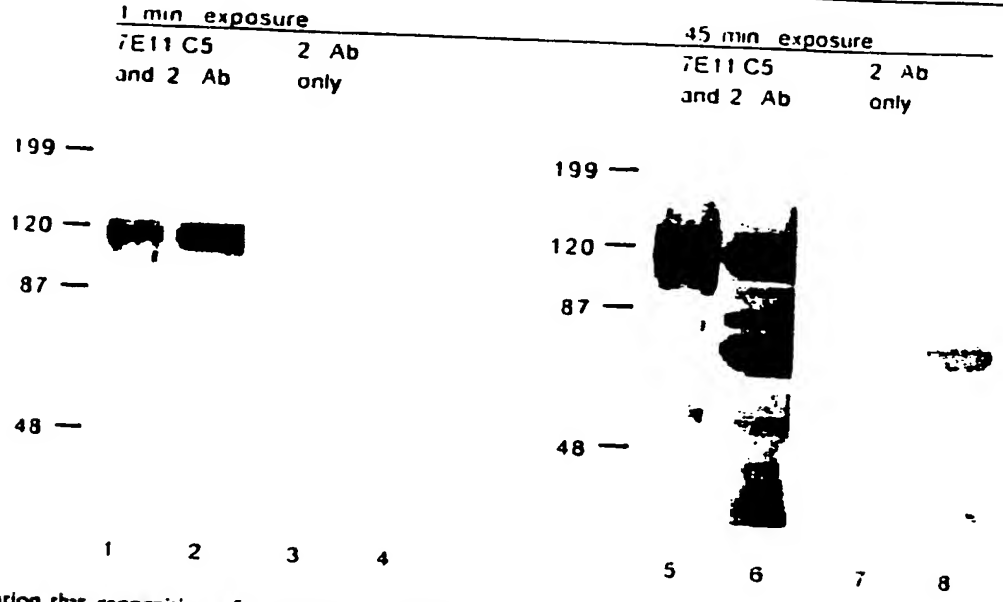


Fig. 5. Demonstration that recognition of a protein in LNCaP lysate (*lanes 1, 5*) and in the serum of cancer patients (*lanes 2, 6*) by 7E11.C5 is specific to the primary antibody 7E11.C5 and not due to nonspecific recognition by secondary antibody used to report primary antibody binding to proteins. LNCaP lysate (0.05 μg protein/well; *lanes 1,3,5,7*) or prostate cancer patient serum (0.44 μg protein/well; *lanes 2,4,6,8*) was separated on SDS gels as previously described and transferred to Immobilon P paper and processed for Western blot as follows. *Lanes 1,2,5,6* were probed with 7E11.C5 monoclonal antibody and then with secondary antibody (i.e., goat antimouse IgG) or with secondary antibody only (*lanes 3,4,7,8*). The film was exposed for either the routine 1 min (*lanes 1–4*) or overexposed for a period of 45 min (*lanes 5–8*). These results demonstrate that the recognition of bands by 7E11.C5 is specific for the primary antibody, and not due to nonspecific binding of secondary or reporting antibody. It should be noted that the same secondary antibody is used with monoclonal 3F5.4G6 as with 7E11.C5, and therefore the 3F5.4G6 monoclonal antibody is specific for PSMA and PSM′ as illustrated in Figure 4, 6, 7 and is not due to nonspecific binding of the secondary antibody to proteins in the lysate.

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